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Original Article

Efficiency of Nested-PCR in Detecting Asymptomatic Cases toward Malaria Elimination Program in an Endemic Area of Iran

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Abstract

Background: The aim of this study was to detect low parasite and asymptomatic malaria infections by means of three malaria diagnostic tests, in a low transmission region of Minab district, Hormozgan Province, southern Iran.

Methods: Blood samples of 200 healthy volunteers from Bagh-e-Malek area were evaluated using microscopic, rapid diagnostic tests (RDT) and nested-PCR to inspect malaria parasite.

Results: The results showed no *Plasmodium* parasite in subjects by means of microscopy and RDT. However, 3 *P. vivax* positive samples (1.5%) were discovered by Nested-PCR while microscopy and RDT missed the cases.

Conclusion: Microscopy as the gold standard method and RDT correctly identified 98.5% of cases, and molecular analysis is sensitive and reliable, especially in the detection of "asymptomatic" infections for active case surveillance. Regarding the existence of asymptomatic malaria in endemic area of Hormozgan, Iran, nested-PCR could be considered as a sensitive tool to interrupt malaria transmission in the country, beside the microscopic and RDT methods.

Introduction

Malaria is still one of the main sources of consideration for public health in many countries including Iran, particularly in south and southeast regions of the country. Deliberate implementation of control measures has resulted in

a substantial reduction of malaria incidence in Iran in recent years (1), therefore malaria cases were reduced from 11,460 cases in 2008 to 3,239 in 2011(Iranian Center for Disease Management and Control, CDMC, unpublished data). In Iran, *P. vivax* is responsible for the majority of malaria cases while *Plasmodium falciparum* accounts for 10-15% of the cases (CDMC, unpublished).

Iran entered the malaria pre-elimination program since 2009 and interventions such as preventing strategies, active case detection and timely appropriate treatment of patients have been performed in all endemic areas of the country.

In order to achieve malaria elimination, which is a global concept to reduce the incidence of locally acquired infection into zero, early recognition of all parasite carriers bearing various disease symptoms and parasite rates is crucial. In this situation, active surveillance by precise laboratory methods in endemic areas provides an appropriate tool for detection of all kinds of infections particularly asymptomatic ones containing low parasite densities (2).

Formerly asymptomatic malaria infections were described in hyper-endemic regions following frequent infective bites and repetitive immune system response toward the parasite attenuating parasitemia to undetectable levels in blood smear (3, 4).

The prevalence of this kind of malaria infection was determined by means of different laboratory techniques in various endemic countries previously (5-9), in parallel with other counties there are a few studies in Iran which assessed asymptomatic malaria prevalence in different low endemic areas of the country (10-12). Based on the controversial results of previous studies which show the fluctuation of asymptomatic infection among the plenty of endemic regions, the importance of regional acquiring of symptomless infection was elucidated as well (10, 11).

Another highly important issue that has to be taking account in asymptomatic malaria investigation is using accurate detection methods to find all carriers, principally who does not have clinical malaria symptoms. This highlights the vitality of evaluating diagnostic method's delicacy for further decisions on malaria elimination interventions.

To date, light microscopy of Giemsa-stained thick and thin blood smears has been remained as the standard laboratory method of malaria diagnosis due to specificity and ability to quantify the level of parasitemia (13). However, the reliance to the results of light microscopy is restricted due to the limitations associated with this method (14). Although Microscopy is the most commonly used method of malaria diagnosis as the gold standard in Iran, chromatographic rapid diagnostic tests (RDTs) are introduced in remote settings, where microscopic diagnosis is either impractical or unfeasible and as a complementary method for confirming the parasite-infected cases.

Besides the mentioned methods, the usefulness of molecular methods such as nested-Polymerase Chain Reaction (PCR) has been emphasized as highly sensitive means of malaria detection, particularly in sub-clinical parasitemia levels (15-17).

The aim of this study was to detect low parasite and asymptomatic malaria infections by means of three malaria diagnostic tests (microscopy, RDT and nested-PCR), in a low transmission region of Bagh-e-Malek in Minab district, Hormozgan Province, southern Iran. The results of this investigation would help further case surveillance strategies in the region and additionally all endemic settings of Iran, as an essential approach toward achieving malaria elimination all over the country.

Material and Methods

Study site and sampling

The present study was performed as a cross-sectional survey in Bagh-e-Malek region, which is located in Minab district, southeast of

Hormozgan Province. Hormozgan is one of the endemic areas in southern Iran, containing about 10.8% of all Iranian malaria patients in 2011 (349 cases reported based on the Iranian CDMC unpublished report) (Fig. 1). Among all over Minab district the higher rate of transmission has been reported from Bagh-e Malek area, therefore this region was selected for this project. Bagh-e-Malek is a lowland area surrounded by mountains, which isolate it from the neighboring regions in Minab, which has a population of 1,451 people. The annual average temperature of Minab is 28 ◦C; pluviometer index of 249 mm and an annual relative humidity of 55.5%.

The total malaria cases in this area were 15 in 2011 (Iranian CDMC), while *Plasmodium Vivax* is the predominant species in Bagh-e Malek and whole province, also the chief anopheles vectors in Minab are *Anopheles stephensi*, *An. culicifacies* and *An. Fluviatilis* (18). Mainly two annual peaks of malaria are seen in this area; which occurs in May and October.

Fig. 1: Iran's map, depicting the study area

A total of 200 healthy volunteers were participated in this study from Bagh-e Malek area (62 males and 138 females). Briefly 3 ml fresh blood sample were collected during July 2011 from the participants after getting informed consent, for further microscopic, RDT and molecular analysis. The study was approved by the Ethics Committee of Hormozgan University of Medical Sciences (approval no. HEC-92-4-1). Inclusion criteria such as lack of having any malaria symptom or receiving antimalarial drug in three weeks prior to the study and having no travel history to other malaria endemic areas in the past three months were considered in sample collection.

Microscopy

Thick and thin blood smears were prepared from each individual according to standard procedure. Thin films fixed using methanol then every slide stained with 10% Giemsa and examined at ×1000 with oil immersion to inspect malaria parasites by a skilled microscopist.

Further thick and thin blood smear preparation were carried out from all participants, 30 days following the sampling date as well as 60 days afterwards, in order to follow up the samples.

RDT

All specimens were tested with the First Response Malaria pLDH/HRP2 Combo assay (Premier Medical Corporation Ltd., Mumbai, India). The kit contains a two line strip precoated with two monoclonal antibodies, one against the pan-specific lactate dehydrogenase, pLDH, of the *Plasmodium* species (*P. falciparum, vivax, malariae, ovale*) and the other antibody was against *P. falciparum* species histidine-rich protein 2 (HRP2). The assay was performed following the manufacturer's instructions. Briefly, 5 µL of whole blood was dispensed onto sample well followed by two drops of assay buffer (60 µl) into developer well. The test control line was observed as an indicator, in order to validate the performance of the test. The results in Control and test lines were appeared within 20 minutes. Finally, differential diagnosis between *P. falciparum* and non-*falciparum* species were interpreted as described previously (19).

Nested-PCR

The molecular detection of *Plasmodium* species was performed using nested-PCR amplification of 18 ssrRNA of the parasite. To achieve this purpose, DNA was extracted from whole blood samples using Promega Kit (Promega, Madison, WI, USA) and stored at -20°C until further analysis with primers and cycling conditions that described previously (11,20).

In brief, 5 µL of extracted DNA was used in the first nest, by use of primers targeting an external region specific to the *Plasmodium* genus followed by second nest using 2 µL of the first nested-PCR product as template to amplify specific *P. vivax* and *P. falciparum* fragments using internal primers. Amplification was performed in a total volume of 25 µL including, 2-5 µL of templates, 250 nM of primers, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 125 µM of each of the four deoxynucleotide triphosphates and 0.4 U of Taq polymerase (Invitrogen, Carlsbad, CA). First and second nests repeated for 25 and 30 cycles, respectively while the annealing temperature was 72 °C for both reactions. Amplicons from the second nest of PCR were electrophoresed on 2% agarose gel and visualized under UV light after staining with ethidium bromide. A sample was considered positive for *P. vivax* and *P. falciparum* if a 120 and 419 base-pair fragment was detected, respectively. Negative and positive controls were run in each reaction.

Results

This investigation was carried out in order to assay the concordance between the outcomes of routine malaria diagnostic techniques with nested-PCR detecting method in finding asymptomatic cases in elimination phase of malaria control. Hence, blood samples and smears were collected from 200 individuals residing in the endemic region of Bagh-e-Malek located in Minab district, Hormozgan province of Iran. All of specimens were evaluated using microscopic, RDT and nested-PCR analysis in Hormozgan Provincial Health Center laboratory. All of participants were healthy individuals and age range was from 3 to 60 years old (mean age 27.2) (Table 1).

Table 1: Details of demographic data and the frequency of asymptomatic infection detected by use of nested-PCR

Study Subjects	Sex		Age Group		Malaria History	
	Male	Female	≤ 15	> 15	Yes	No
No. (%)	62 (31)	138 (69)	55 (27.5)	145 (72.5)	25 (12.5)	175 (87.5)
Total	200 (100)		200 (100)		200 (100)	
PCR Positive No. (%)	0	3 (100)	1(33.3)	2 (66.6)	1(33.3)	2 (66.6)
Total	3 (1.5)		3 (1.5)		3 (1.5)	

Microscopic examination

The entire 200 thick and thin Giemsa-stained blood smears were inspected to detect *Plasmodium* parasite by well-trained microscopist in Hormozgan Provincial Health Center laboratory and no *Plasmodium* parasite were detected in any examined sample. Additionally none of samples was diagnosed to have any malaria parasite during the 60-days follow up protocol, while all of samples remained symptomless.

RDT interpretation

RDTs were tested as a complementary analysis for all of 200 samples using monoclonal antibody coated sticks. No positive result was discovered for either *P. falciparum* and/or non-*falciparum* species in the performed tests.

Asymptomatic malaria detection using Nested-PCR

In this cross sectional study, nested-PCR amplification was performed as a sensitive and specific method to find parasite genome in extracted DNA from asymptomatic samples. Interestingly 3 *P. vivax* positive samples (1.5%) were discovered by nested-PCR using specific primers, although they were considered uninfected using microscopic and RDT tests (Table 1). These cases were considered as asymptomatic malaria patients since they remained symptomless in follow up evaluations (i.e. thick blood smear and RDT) prior to treatment. All positive cases were female and only one of them had a malaria history 20 years prior to the time of this investigation. Moreover, no *Plasmodium falciparum* infection was detected by this technique for all examined samples. All infected subjects were treated after diagnosis by standard anti-malarial drugs according CDMC of Iran protocol and re-evaluated after treatment by means of molecular method.

Discussion

In the elimination phase of malaria control, monitoring all cases particularly hidden parasites is of major importance. This highlights choosing of an acceptable detecting method, which should be, applied in all incoming asymptomatic investigation afterwards in order to detect every single carrier which continue disease transmission in the population (21).

Currently in malaria endemic areas of Iran, microscopic and RDT assays are used for active case surveillance, although these methods could detect nearly all of symptomatic infections, in case of asymptomatic and mixed infections a more reliable technique would be helpful especially in elimination program as a public health concern in the country.

Although, microscopy is still the diagnosis gold standard, limitations such as requiring well skilled microscopists, technical equipment maintenance, discrepancy in microscopist training and insufficient quality control are associated with this method (22). Although expert microscopy could theoretically detect 10 p/μL parasitemia, average microscopy has proven to be unable in detecting lower than 100 p/μL parasitemia (23). However, it should be mentioned that microscopy is the primary and easiest to establish method in the field situation.

Alternatively, despite the simplicity and being easy to use without particular training which highlight the role of RDTs in malaria detection, they may have some disadvantages; for instance, the intensity of test band is subject to amount of antigen particularly low parasite densities, and this may lead to reader variation in test results. Furthermore, the short shelf life of RDTs emphasizes requiring of proficient transportation, storage and distribution systems, additionally, diagnose of asymptomatic malaria infection realized to be unreliable using RDTs (23-25).

In addition to the importance of microscopy and RDT, mainly in field settings, in a number of recent investigations molecular methods were nominated as valuable and robust approaches particularly in detecting sub-clinical parasitaemia i.e. asymptomatic carriers, mixed infections and confirmation of *Plasmodium* species in low-transmission areas (26-29).

However, regarding PCR expenses and equipment requirements in contrast to usual diagnosis methods, the performance of nested-PCR in identifying asymptomatic carriers was compared to conventional microscopic and RDTs in symptomless population of Bagh-e-Malek, a malaria endemic region, in southern Iran. Interestingly nested-PCR found three *P. vivax* carriers while microscopy and RDT missed the cases, one of the patients was a 12-year-old symptomless girl, who experienced a period of fever in the year prior to the study, and her disease has not been diagnosed since her thick and thin blood smears analyzed negative by light microscopy. The blood films

of this patient remained negative even in re-sampling after identifying a positive PCR result.

Conclusion

The current results emphasize the importance of using molecular methods in order to interrupt malaria transmission in Iran besides microscopy and RDTs as routine methods. It should be noted that toward eliminating malaria from the country and prevention of re-introduction, all the three types of malaria detection tools (microscopy, RDT and molecular methods such as nested-PCR) should be used to improve sensitivity, specificity and reliability for active case surveillance in endemic settings.

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